

Analytical Validation of Molecular Genetic Tests Intended for the Analysis of the Human Genome

1. Introduction

The analysis of the human genome using molecular genetic tests has become an integral part of the diagnosis of many diseases. Since increased turnaround of patients between individual facilities, whether on the national level or even abroad, is now expected, there will be an growing requirement for mutual compatibility of results from different facilities utilising these tests. Sufficient validation should therefore become an essential part of the tests to be performed. Validation of molecular genetic tests is one of the major requirements stipulated in the ISO 17025 and ISO 15189 standards. According to these standards, validation is a confirmation through tests and provision of objective proof that the individual requirements for specific objectives have been met. Nevertheless, the rules for the performance of validations of molecular genetic tests for the analysis of the human genome have not been strictly determined yet. For this reason, the present document has been prepared as a recommendation for validation of molecular genetic tests utilised in the analysis of the human genome. Validation of each specific test must be based on analysis of the relevance and importance of parameters evaluated for the given test and purpose of use (“fit-for-purpose”).

This recommendation pertains to the analytical validation of molecular genetic tests only. In addition to analytical validation, clinical validity of the results issued should always be assessed separately in cooperation with clinical facilities and based on current scientific literature. This clinical validation should include verification of the relationship between the analyte to be tested and the solution of the respective diagnostic problem.

With respect to diagnoses for which an existing recommendation is available as part of the Guidelines for Good Laboratory Practice/**Best practice**

(http://www.uhkt.cz/lab_a_vysetreni/hr_lab_dna_diag/best_lab_practice), **the recommended test is deemed to be standardized** and may be therefore validated (verified) using an abbreviated procedure; see articles 2.b.2. and 3.b.2.

Currently, an **established system of internal quality control** and where available **participation in external quality assessment** (EQA/PT http://www.uhkt.cz/lab_a_vysetreni/hr_lab_dna_diag/koord_centrum) are essential requirements for any molecular genetic test to be performed. The system of internal quality control schemes must include systematic **use of both positive and negative controls**, in the case of quantitative tests for samples with known and verified quantity of the parameter tested. For **tests employing PCR**, use of **NTC** (no template control) is required to rule out possible contamination (the sample contains no added DNA).

Molecular genetic test validation, in its broadest sense, has to comprise all steps of the test used, including all preparatory procedures, but not limited to DNA/RNA isolation, as well as validation of the devices in use.

Where software analysis is one of the steps of a validated molecular genetic test, the computer program used must also be validated.

Robustness of the molecular genetic test for the respective parameters should be examined when changing the batch of critical components.

Validation is also strictly bound to the quality and source of both the samples to be tested and control samples used for comparison.

Validation protocol, which must include any and all data on the ways of performance and evaluation, must be an integral part of any validation. No validation process is valid unless the standard operating procedure used is compliant with the procedure used during the validation.

2. Validation and verification of qualitative molecular genetic tests

2.a. Full validation of the qualitative molecular genetic tests developed in the laboratory

Required validation parameters:

- Determination of **specificity and sensitivity**, i.e., determination of all variants (mutations/polymorphisms) of the parameter tested (gene/nucleotide sequence), which are determined using this molecular genetic test, and testing of **negative control**, i.e. DNA/RNA not containing the trait examined to exclude false positivity.
 - **Specificity** is the probability of a negative result of the test in the absence of the examined variant of the trait tested. It is expressed as a ratio between true negativity (*TN*) and the sum of true negativity and false positivity (*FP*) according to the formula as follows:

$$\text{TN}/(\text{TN}+\text{FP})$$

The resulting value is expressed as a relative number, the value of which ranges between 0 and 1 (ideally it approaches 1)

- **Sensitivity** is the probability of a positive result of the test in the presence of the examined variant of the trait tested. It is expressed as a ratio between true positivity (*TP*) and the sum of true positivity and false negativity (*FN*) according to the formula as follows:

$$\text{TP}/(\text{TP}+\text{FN})$$

The resulting value is expressed as a relative number, the value of which ranges between 0 and 1 (ideally it approaches 1)

Specificity and sensitivity are determined using certified reference material (CRM), if available. Where the CRM is not available, one should have his/her own reference material, the positivity/negativity of which has been verified by another independent

test. Samples from external quality control, wherein the results have already been confirmed, may also be used as reference material.

- Testing of **repeatability**, i.e., mutual comparison of results in a single series – parallel within a single day by the same employee (at least 10 times per sample is recommended).
- Testing of **reproducibility**, i.e., mutual comparison between the series – on different days, by different employees – (at least 10 times per sample is recommended).
- Determination of the **detection limit**, i.e., minimum quantity of the trait (analyte) tested/DNA/RNA/cells*, which is/are necessary to perform the test clearly and with the required specificity and sensitivity using the CRM, if available. Where the CRM is not available, one should have his/her own reference material, in which the quantity of the trait (analyte) tested/DNA/RNA/cells* have been determined by another independent test. Samples from external quality control, in which the results have already been confirmed, may also be used as reference material.
- Determination of **robustness**, which is used to examine the sensitivity of the test to deviations, if any, from optimal conditions during the performance thereof (for example different thermocyclers, different polymerases, different restrictases, including different quantities of input components, and so on.)
Example: We should examine the determined specificity and sensitivity of the test when changing the thermal cyclers.
- Determination of **identification uncertainty** (only for certain qualitative tests – such as determination of alleles in repetitive sequences).

* To select a suitable parameter depending on the test used and purpose of use.

2.b. Validation of the adopted qualitative molecular genetic tests

2.b.1. If the test has not been standardized,

a full validation of this test should be performed (see 2.a). Tests adopted from the literature cannot be deemed as being standardized.

Note: Standardized molecular genetic tests are those developed by a standardization/normalization authority or other renowned organization, the tests of which are generally accepted in the respective industry/by a professional society. (MPA 30-04-05 Flexible range of accreditation in the area of testing laboratories).

In this respect, the Society for Medical Genetics (SLG, www.slg.cz) acts as a professional organization in the area of genetics.

2.b.2. If the test has been standardized and used exactly in accordance with the instructions for use,

then we should perform a shortened validation procedure (verification) using the following parameters:

- Testing of **repeatability**, i.e., mutual comparison of results in a single series – parallel within a single day by the same employee (at least 6 times per sample is recommended).
- Testing of **reproducibility**, i.e., mutual comparison between the series – on different days, by different employees – (at least 6 times per sample is recommended).
- Determination of the **detection limit**, i.e., minimum quantity of the trait (analyte) tested/DNA/RNA/cells*, which is/are necessary to perform the test clearly and with the required specificity and sensitivity using the CRM, if available. Where the CRM is not available, one should have his/her own reference material, in which the quantity of the trait (analyte) tested/DNA/RNA/cells* have been determined by another independent test. Samples from external quality control, in which the results have been already confirmed, may also be used as reference material.
- Determination of **robustness**, which is used to examine the sensitivity of the test to deviations, if any, from optimal conditions during the performance thereof (for example different thermocyclers, different polymerases, different restrictases, including different quantities of input components, and so on), especially if we cannot follow the original guideline (see the note below).
- Determination of **identification uncertainty** (only for certain qualitative tests – such as determination of alleles in repetitive sequences). It can be adopted from this protocol if so specified in the standardization protocol.

* To select a suitable parameter depending on the test used and purpose of use.

Note: If not used exactly in accordance with the recommendations of the guidelines (http://www.uhkt.cz/lab_a_vysetreni/hr_lab_dna_diag/best_lab_practice), the molecular genetic test cannot be deemed standardized and a full validation thereof should be performed (see 2.a.).

2.c. Validation of the qualitative molecular genetic tests using commercially produced IVD diagnostic kits with the CE mark.

When using the IVD diagnostic kits with the CE mark without any changes to the provided instructions for use, a shortened validation can be carried out, in other words, verification to confirm that the diagnostic kit used in a laboratory shows identical values to those specified by the manufacturer in a provided validation protocol and/or package leaflet. The following rules shall apply for this type of verification:

- The diagnostic kit should be used exactly in accordance with the manufacturer's instructions for use without any changes.
- The diagnostic kit should be used only for the purposes as declared by the manufacturer.
- The verification procedure is identical to that used for the verification of standardized molecular genetic tests, see article 2.b.2.

Note: Should the diagnostic kit not be used exactly in accordance with the instructions for use, it should be validated (see article 2.a.) for those parameters that may be influenced by a change to the procedure/protocol.

If the validation protocol with the required parameters is not provided, the manufacturer/distributor should be requested to correct this failure. *Pursuant to the IVD directive, or Decree of the Government No. 453/2004 Coll., the manufacturer is obliged to indicate selected traits in the package leaflet.*

3. Validation and verification of quantitative molecular genetic tests

3.a. Full validation of the quantitative molecular genetic tests developed in the laboratory

- Determination of **specificity and sensitivity**, i.e., determination of all variants of the parameter tested, which are determined using this molecular genetic test, and testing of **negative control**, i.e., DNA/RNA not containing the trait examined to rule out false positivity.
 - **Specificity** is expressed as a ratio between true negativity (*TN*) and the sum of true negativity and false positivity (*FP*) according to the formula as follows:

$$\text{TN}/(\text{TN}+\text{FP})$$

- **Sensitivity** is expressed as a ratio between true positivity (*TP*) and the sum of true positivity and false negativity (*FN*) according to the formula as follows:

$$\text{TP}/(\text{TP}+\text{FN})$$

Specificity and sensitivity is determined using CRM, if available.

Where the CRM is not available, one should have his/her own reference material, the positivity/negativity of which has been verified by another independent test.

Samples from external quality control in which the results have already been confirmed may also be used as reference material.

- Testing of **repeatability**, i.e., mutual comparison of results in a single series – parallel within a single day by the same employee (at least 5 x 1 sample in three concentrations at the outer limits and in the middle of the detection range).

- Testing of **reproducibility**, i.e., mutual comparison between the series – on different days, by different employees (at least 5 times per sample in three concentrations is recommended at the outer limits and in the middle of the detection range).
- From the values obtained during the testing of repeatability and reproducibility, to determine the **precision** expressed as a **value of measurement uncertainty** using a suitable parameter, for example through **determination of the standard deviation** or **coefficient of variation**.

The value of measurement uncertainty should take into account all other components that may have a substantial influence on it (for example uncertainties of the meters used, systematic deviations of the values determined, ...) see Suchánek M., Friedecký B., Kratochvíla J., Budina M., Bartoš V.: Doporučení pro určení odhadů nejistot výsledků měření/klinických testů v klinických laboratořích [Recommendations for estimating the uncertainty of results of measurements/clinical tests in clinical laboratories].

- Determination of the **detection range**, i.e., minimum quantity/concentration of the trait (analyte) tested/DNA/RNA/cells*, within which the test works with required precision when using the CRM with known concentration/quantity of the trait (analyte) tested/DNA/RNA/cells*, if available. Where the CRM is not available, one should have his/her own reference material, in which the concentration/quantity of the trait (analyte) tested/DNA/RNA/cells* has been determined by another independent test. Samples from external quality control, in which the results have already been confirmed, may also be used as reference material.

* To select a suitable parameter depending on the test used and purpose of use

3.b. Validation of the adopted quantitative molecular genetic tests

3.b.1. If the test has not been standardized,

a full validation of this test should be performed (see 3.a). Tests adopted from the literature cannot be deemed as being standardized.

3.b.2. If the test has been standardized and used exactly in accordance with the instructions for use,

then we should perform a shortened validation procedure (verification) using the following parameters:

- Testing of **repeatability**, i.e., mutual comparison of results in a single series – parallel within a single day by the same employee (at least 3 times per sample is recommended in three concentrations at the outer limits and in the middle of the detection range).

- Testing of **reproducibility**, i.e., mutual comparison between the series – on different days, by different employees (at least 3 times per sample is recommended in three concentrations at the outer limits and in the middle of the detection range).
- From the values obtained during the testing of repeatability and reproducibility, to determine the **precision** expressed as a **value of measurement uncertainty** using a suitable parameter, for example through **determination of the standard deviation** or **coefficient of variation**.

The value of measurement uncertainty should take into account all other components that may have a substantial influence on it, especially the uncertainty specified in the description of a standardized test, see Suchánek M., Friedecký B., Kratochvíla J., Budina M., Bartoš V.: Doporučení pro určení odhadů nejistot výsledků měření/klinických testů v klinických laboratořích [Recommendations for estimating the uncertainty of results of measurements/clinical tests in clinical laboratories]

- Determination of the **detection range**, i.e., minimum quantity/concentration of the trait (analyte) tested/DNA/RNA/cells*, within which the test works with a required precision when using the CRM with known concentration/quantity of the trait (analyte) tested/DNA/RNA/cells*, if available. Where the CRM is not available, one should have his/her own reference material, in which the concentration/quantity of the trait (analyte) tested/DNA/RNA/cells* has been determined by another independent test. Samples from external quality control, in which the results have already been confirmed, may also be used as reference material.

* To select a suitable parameter depending on the test used and purpose of use.

Note: If not used exactly in accordance with the recommendations of the guidelines (http://www.uhkt.cz/lab_a_vysetreni/hr_lab_dna_diag/best_lab_practice), the molecular genetic test cannot be deemed standardized and a full validation thereof should be performed (see 3.a.).

3.c. Validation of the quantitative molecular genetic tests using commercially produced IVD diagnostic kits with the CE mark

When using the IVD diagnostic kits with the CE mark without any changes to the provided instructions for use, a shortened validation can be carried out, in other words, verification to confirm that the diagnostic kit used in a laboratory shows identical values to those specified by the manufacturer in a provided validation protocol and/or package leaflet. The following rules shall apply for this type of verification:

- The diagnostic kit should be used exactly in accordance with the manufacturer's instructions for use without any changes
- The diagnostic kit should be used only for the purposes as declared by the manufacturer.

- The verification procedure is identical to that used for the verification of standardized molecular genetic tests, see article 3.b.2.

Note: Should the diagnostic kit not be used exactly in accordance with the instructions for use, it should be validated (see article 3.a.) for those parameters that may be influenced by a change to the procedure.

If the validation protocol with the required parameters is not provided, the manufacturer/seller should be requested to correct this failure. *Pursuant to the IVD directive, or Decree of the Government No. 453/2004 Coll.*, the manufacturer is obliged to indicate selected traits in the package leaflet.

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